The Protein Factory

L-Aspartate oxidase from *Sulfolobus tokodaii*: immobilization studies

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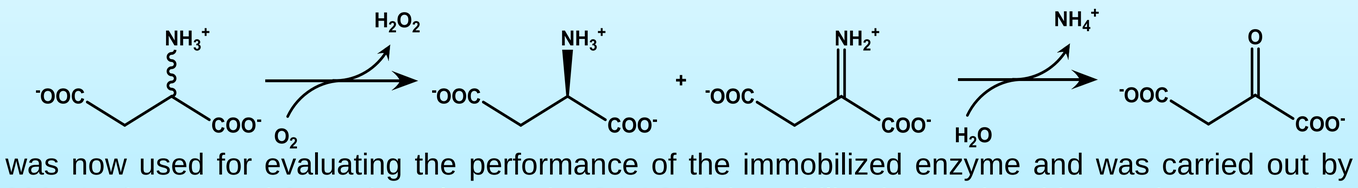
Introduction

L-Amino acid oxidases (LAAOs, EC 1.4.3.2) catalyze the stereospecific oxidative deamination of L-amino acids to α-keto acids, ammonia and hydrogen peroxide.[1,2] In our labs L-aspartate oxidase from the thermophilic archaea *Sulfolobus tokodaii* (StLASPO, EC 1.4.3.16) has been efficiently produced as recombinant protein in *E. coli* in the active form as holoenzyme[3] and fully characterized.
The enzyme is active only on L-aspartate and on L-asparagine and it's absolutely stereoselective. StLASPO shows a remarkable stability: up to 80 °C in the 7 – 10 pH range. Plotting the activity (on L-aspartate) against temperature in the 25 – 95 °C range, an increase with no evidence for any plateau or decrease up to 80 °C was apparent. The activity of the enzyme as a function of pH shows a bell-shaped behavior: it is maximal at about pH 10.

Kinetic resolution of D,L-aspartate

In a previous study StLASPO was used for the resolution of a 50 mM solution of D,L-aspartate, and the full resolution was reached in 4 hours in the 37-70 °C and pH 10-11 range using 0.3 U StLASPO/mL.



The kinetic resolution of D,L-aspartate was now used for evaluating the performance of the immobilized enzyme and was carried out by adding 0.5 mL of a 50 mM D,L-aspartic solution (in water, adjusted to pH 10.0), 200 µL of water and 2 µL of catalase to the StLASPO immobilized on resin. This reaction was performed at 70 °C on a thermomixer set to 600 rpm.
For CLEA preparation the assay was performed by adding 0.5 mL of a 50 mM D,L-aspartic solution in "magic buffer" (160 mM Tris–HCl, 160 mM Na_2CO_3, 160 mM H_3PO_4, 0.65 mM potassium chloride and 1% glycerol) padjusted to the desired pH (8, 9 or 10) and 1 µL of catalase to the CLEA-StLASPO preparation (1.12 U); the reaction was performed at 70 °C on a thermomixer set to 600 rpm.

Immobilization on Relizyme™ HA403/SR

Cycle number	Time (h)	e.e. (%)
1	1	100
2	1	100
3	1	100
4	1	87
	2	100
5	1	77
	2	100

The resin Relizyme™ HA 403/SR (250 mg), which has amino moieties, was treated with 10 mL of 0.125% glutaraldehyde solution in water for 2.5 h on a rotating device at 18 °C. The glutaraldehyde solution was then removed and the resin was washed with 0.1 M phosphate buffer at pH 7.5.

After that, 1 mL of StLASPO (2 U) was added to the resin, with 1 mL of 0.1 M phosphate buffer at pH 7.5 and the mixture was incubated on a rotatory shaker for 24 h at 18 °C.

StLASPO was immobilized with a full yield in terms of activity.
The resin was then washed with 50 mM phosphate buffer at pH 7.5, and stored at 4 °C in the same conditions.

The immobilized enzyme showed an activity of 8 U/g matrix

The influence of D,L-aspartate concentration on enzymatic (2 U/118 mg resin) resolution was also evaluated, by adding 0.5 mL of a D,L-aspartic solution at different concentration (from 50 to 500 mM) at pH 10.0.

	L-aspartate conversion (%)								
Final D,L- Asp (mM)	Time (h)								
	0.5	1	2	3	4	5	7	21	42
35	100	-	-	-	-	-	-	-	-
70	62	100	-	-	-	-	-	-	-
110	12	36	100	-	-	-	-	-	-
140	-	-	-	67	100	-	-	-	-
180	-	-	-	-	53	69	84	-	-
360	-	-	-	-	17	21	21	21	21

Analytical

At predetermined intervals, 25 µL aliquots were withdrawn from the reaction mixture and diluted in 100 µL of distilled water. 10 µL of this solution were derivatized with 25 µL of OPA-NAC reagent, diluted with 25 µL of 50 mM eluent and analyzed by HPLC cromatography (column Gemini 5µ C18 Phenomenex; eluent: 50 mM sodium acetate buffer pH 5.2 / MeOH 90/10; flux of 0.8 mL/min; detection at 340 nm).

OPA-NAC reagent is a basic aqueous-methanolic solution of ortho-phthalaldehyde and N-acetylcysteine which are able to react with free amine giving a fluorescent derivate.

Other supports tested

Also other solid supports were evaluated:
Sepabeads EC-EP/A, Relizyme™ EP113, Relizyme™ HA403, Relizyme™ HA113, Eupergit® C, IDA-3 Sepharose® yielded poor results in term of immobilization except for IDA-3 resin: this matrix showed a high immobilization yield but the enzyme was leached out due to the non-covalent interaction with the matrix.

Immobilization on resin SEPABEADS EC-EP/S

Cycle number	Time (h)	e.e. (%)
1	3	68
	4	100
2	2	66
	4	100
3	2	65
	4	100
4	2	59
	4	81
	6	100
5	2	28
	4	45
	6	73
	8	80
	10	100

One unit of pure StLASPO was added to 60 mg of Sepabeads EC-EP/S, with oxirane moieties, and to 0.3 mL of 1.25 M KH_2PO_4 buffer at pH 8.0.

The mixture was incubated on a rotatory shaker for 18 h at 25 °C: approximately 50% of the enzymatic activity was present in solution suggesting that half of the starting enzyme has been immobilized.

This immobilized StLASPO preparation yielded full conversion of L-isomer of aspartate in 4 hours for 3 cycles. For the following cycles, the full resolution was obtained in a longer time, requiring up to 10 hours in the fifth cycle.

Immobilization as CLEA

The precipitation of StLASPO as a CLEA[4] was conducted by adding to 0.5 mL of enzyme solution (2.24 U/mL), 4.5 mL of a 60% w/v $(NH_4)_2SO_4$ solution, in 0.1 M phosphate buffer at pH 7.5 and 20 µL of glutaraldehyde.

The mixture was incubated on a rotatory shaker for 2.5 h at room temperature: no residual enzymatic activity was detected in the solution.CLEA-StLASPO was then washed twice with 0.1 M phosphate buffer at pH 7.5 and was stored at 4 °C in 0.05 M phosphate buffer at pH 7.5.

Before each cycle, the CLEA-StLASPO preparation was incubated with 1 µL of FAD for 30 min in 0.1 M phosphate buffer at pH 7.5.

Cycle number	Reaction time (h)	e.e. (%)[a] pH 8	e.e. (%)[a] pH 9	e.e. (%)[a] pH 10
1	2	49	32	53
	4	87	100	100
	6	100	-	-
2	2	49	63	66
	4	97	100	100
	6	100	-	-
3	2	69	69	64
	4	73	100	100
	6	100	-	-
4	2	88	54	42
	4	100	100	68
	6	-	-	100
5	2	91	91	40
	4	100	100	69
	6	-	-	95
	7	-	-	100
6	2	0	0	31
	4	0	0	56
	6	0	0	80
	8	0	0	100

In the first cycle, the conversion was fully reached at all pH values tested, in a comparatively shorter time at highest pH. On the contrary, starting from the fourth cycle, the full oxidation of L-aspartate was obtained in a shorter time at pH 8 and 9.

Conclusions

Due to the very interesting properties of this enzyme, we propose the immobilized StLASPO as an attractive tool for biotechnological applications. We recognize that CLEA technique or Relizyme™ HA403/SR as solid support represent the best conditions for StLASPO immobilization: this step will allow to improve the stability as well as enzyme reusability.

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